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Treatment of Locally Recurrent Breast Carcinoma

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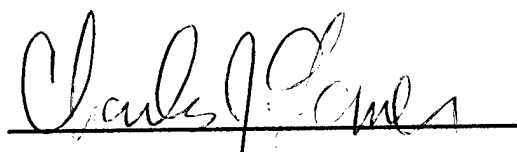
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## 5. INTRODUCTION

Photodynamic therapy (PDT) is a developing therapeutic modality which continues to show promise in the clinical treatment of cancer, including locally recurrent breast carcinoma. Our application is directly related to using novel molecular technologies to improve the effectiveness of PDT for treating locally recurrent breast cancers. In PDT, properties of photosensitizer localization in tumor tissue and photochemical generation of reactive oxygen species are combined with precise delivery of laser generated light to produce a procedure offering local tumoricidal activity. We have demonstrated that PDT mediated oxidative stress is a strong transcriptional inducer of stress proteins belonging to the heat shock protein (hsp) and glucose regulated protein (grp) families. We have also shown that the hsp and grp promoters can drive inducible expression of heterologous genes following PDT mediated oxidative stress. Inducible expression and function of p53 as well as inducible expression, secretion and biological activity of TNF- $\alpha$  have been documented in human tumor cells. We have also demonstrated PDT inducible expression of the suicide gene HSV-thymidine kinase and enhanced tumoricidal action when PDT is combined with inducible HSV-TK gene therapy. These studies address a critical problem associated with improving treatments for locally recurrent breast cancer using new approaches which will minimize systemic toxicity and maximize a patient's quality of life.

## 6. BODY

There were two specific tasks approved in our Statement of Work. Task #1 was to transfect human breast cancer cells with our Photodynamic Therapy (PDT) inducible expression plasmids and evaluate PDT as a molecular switch for cytotoxic gene expression. This task was further broken down to involve obtaining stably transfected cell lines, evaluation of the expression of suicide genes, and the production of tumors in mice containing cells stably transfected with the inducible gene expression vectors. This task was scheduled to take place during the first 18 months of the project. Specific Task #2 was to determine the tumoricidal efficacy of PDT in mice implanted with breast cancer cells containing the PDT inducible expression vectors. We have been fortunate to have completed most of the specific aims in Task #1 and, therefore, we have been able to start on Task #2. We have used a mouse mammary carcinoma cell line instead of a human mammary carcinoma cell line for initial work because this has allowed us to perform a much larger number of studies. We have also used a PDT inducible grp promoter for the study since we see enhanced inducible expression. We have also used a thymidine kinase suicide gene, and we see excellent tumoricidal action with this system.

Our studies currently show that inducible expression of the herpes simplex virus (HSV) thymidine kinase gene is observed in TSA mouse mammary carcinoma cell lines stably transfected with a G1NaGrpTK retroviral inducible gene expression vector. This inducible expression vector is selectable by G418 and has been stably transduced into TSA mouse mammary carcinoma cells. Activation of the grp promoter in this vector leads to expression of the herpes simplex virus thymidine kinase gene. Cell lysates of the stably transfected cells were assayed for thymidine kinase levels using Western immunoblot analysis. Non-transduced parental cells do not exhibit TK thymidine kinase expression. Transduced cells which served as non-treated controls exhibited basal levels and transduced cells exposed to PDT showed significant expression of thymidine kinase.

With positive proof of principal for in-vitro PDT acting as a molecular switch for inducible expression of thymidine kinase controlled by the grp promoter, we have moved to our next task involving preliminary in-vivo experiments. Here we are asking if our PDT inducible molecular switch functions in mammary tumors growing in mice. The advantage of using the TSA cell line is that we do not need to use the very expensive athymic mice for our studies and, therefore, larger numbers of animals with enhanced statistical significance can be obtained with the funds available for this study. Inducible expression of the HSV thymidine kinase is observed in the TSA tumors following in-vivo PDT treatment. Transduced TSA mouse mammary carcinoma cells were injected subcutaneously in the hind right flank of Balb-C mice. Localized PDT was performed when the resulting tumors

reached 6 mm in diameter. Tissue samples were collected 12 hours after PDT treatment and assayed for thymidine kinase expression by Western immunoblot analysis. Our results show significant selective expression of thymidine kinase within treated tumor tissues.

Since we have positively demonstrated proof of principal that PDT can induce HSV-TK expression in tumors, we next set out to examine whether this expression can actually modulate breast cancer treatment response. Our results show that inducible gene therapy using the grp promoter to drive expression of the HSV-TK suicide gene does enhance tumorcidal action of PDT. Treatment of TSA G1NaGrpTK tumors with a combination of PDT and systemic gancyclovir (GCV) resulted in increased tumor cures compared to PDT treatments alone. Balb-C mice with 6 mm diameter tumors received either PDT treatment plus gancyclovir (10 daily injections of GCV, 100 mg/kg per dose) beginning one hour prior to a single PDT treatment (200 J/cm sq), or PDT alone, or gancyclovir alone. Mice were monitored for tumor recurrences three times per week for 40 days. PDT treatments alone produced 50% tumor cures, while the PDT plus gancyclovir produced 100% tumor cures. Gancyclovir by itself resulted in no tumor cures.

We believe this initial work provides strong proof of principal regarding PDT activated molecular switches for the treatment of breast cancer. Additional studies will now move on forward utilizing the human breast cancer cell lines and more selective PDT treatments.

## **7. KEY RESEARCH ACCOMPLISHMENTS**

- Stable transfection of mammary carcinoma cells containing a stress inducible herpes simplex virus thymidine kinase gene.
- Inducible expression of thymidine kinase following Photodynamic therapy.
- Inducible expression of thymidine kinase in mammary carcinoma cells transplanted into mice.
- Enhanced efficacy of Photodynamic therapy when combined with inducible gene therapy.

## **8. REPORTABLE OUTCOMES**

- Manuscripts

Luna, M.C., Ferrario, A., Wong, S., Fisher, A.M., Rucker, N., Gomer, C.J.: Photodynamic therapy mediated oxidative stress as a molecular switch for the temporal expression of genes ligated to the human heat shock promoter. *Cancer Res* 60:1637-1644, 2000.

- Patents and Licenses

None

- Degrees Obtained

None

- Development of Cell Lines, Tissues, or Serum Repositories

None

- Databases

None

- Funding Applied For

None

- Employment and Research Opportunities

None

## 9. CONCLUSIONS

During the first year of this research project we have been successful in obtaining proof of principal that Photodynamic Therapy (PDT) inducible expression of therapeutic genes is both possible in breast cancer cells grown in culture as well as breast cancer cells transplanted into mice. We have further shown that the inducible expression of the thymidine kinase gene when combined with PDT and gancyclovir offers an enhanced effectiveness in treating mammary carcinomas. The information that we have obtained so far, as it relates to a scientific or medical product, indicates that laser mediated PDT can be an efficient and selective molecular switch for activation of therapeutic genes. Inducible gene therapy has a number of advantages over constitutive gene expression. The ability to control both the temporal and spatial activation of genes is of significant benefit especially when toxic gene products are being produced. Therefore, the ability to provide a molecular means to selectively express genes is of importance.

## 10. REFERENCES

None

## 11. APPENDICES

Luna, M.C., Ferrario, A., Wong, S., Fisher, A.M., Rucker, N., Gomer, C.J.: Photodynamic therapy mediated oxidative stress as a molecular switch for the temporal expression of genes ligated to the human heat shock promoter. *Cancer Res* 60:1637-1644, 2000.

# Photodynamic Therapy-mediated Oxidative Stress as a Molecular Switch for the Temporal Expression of Genes Ligated to the Human Heat Shock Promoter<sup>1</sup>

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## ABSTRACT

Oxidative stress associated with photodynamic therapy (PDT) is a transcriptional inducer of genes encoding stress proteins, including those belonging to the heat shock protein (hsp) family. The efficiency of PDT to function as a molecular switch by initiating expression of heterologous genes ligated to the human hsp promoter was examined in the present study. Selective and temporal reporter gene expression was documented after PDT in mouse radiation-induced fibrosarcoma cells stably transfected with recombinant vectors containing an hsp promoter ligated to either the *lac-z* or *CAT* reporter genes and in transfected radiation-induced fibrosarcoma tumors grown in C3H mice. Hyperthermia treatments were included as a positive control for all experiments. Expression vectors containing either human *p53* or tumor necrosis factor (*TNF*)- $\alpha$  cDNA under the control of an hsp promoter were also constructed and evaluated. A *p53* null and *TNF*- $\alpha$ -resistant human ovarian carcinoma (SKOV-3) cell line was stably transfected with either the *p53* or *TNF*- $\alpha$  constructs. Inducible expression and function of *p53* as well as inducible expression, secretion, and biological activity of *TNF*- $\alpha$  were documented after PDT or hyperthermia in transfected SKOV cells. These results demonstrate that PDT-mediated oxidative stress can function as a molecular switch for the selective and temporal expression of heterologous genes in tumor cells containing expression vectors under the control of an hsp promoter.

## INTRODUCTION

PDT<sup>3</sup> is in clinical trials for the treatment of a variety of solid tumors (1). The porphyrin photosensitizer, PH, recently received FDA approval for PDT treatment of esophageal and endobronchial carcinoma (2). This procedure is also being evaluated in the management of nonmalignant disorders, such as age-related macular degeneration and psoriasis (3). PDT-mediated cytotoxicity relies on the localized photochemical generation of reactive oxygen species, including singlet oxygen (1). This leads to a rapid tumoricidal response mediated by both direct tumor cell toxicity and photodamage to the involved microvasculature (4). A growing number of second generation photosensitizers are also undergoing clinical evaluation (2). These new compounds exhibit properties comparable or superior to PH, including chemical purity, increased photon absorption at longer wavelength, improved tumor tissue retention, rapid clearance from surrounding normal tissues, high quantum yields of reactive oxygen species, and

minimal dark toxicity (5). One such photosensitizer, NPe6, is a water-soluble chlorin involved in Phase I and II clinical trials (6, 7). Direct tumor cytotoxicity and vascular stasis are induced by NPe6-mediated PDT (8). The most effective *in vivo* responses occur when a short time interval (<6 h) is used between NPe6 administration and light treatment (9).

In addition to the development of new photosensitizers, continued improvements in clinical PDT will come from the translation of information generated from studies examining basic mechanisms of this procedure. Biochemical analysis indicates a variety of subcellular PDT targets, including the mitochondria, plasma membrane, and lysosomes (1, 2, 10, 11). Apoptotic and necrotic pathways are both involved in PDT-mediated cell death (2, 12). An assortment of early response genes, genes associated with signal transduction pathways and cytokine expression, as well as stress response genes are activated by PDT (13-19). Stress proteins classified as HSPs are expressed following PDT, and this response is at the level of transcription (18, 19). HSPs are highly conserved throughout evolution and function as molecular chaperones of nascent proteins (20). HSPs are also involved in protecting cells from stress by binding to denatured proteins and assisting in proper refolding (21). Transcriptional regulation of heat shock gene expression involves HSF binding to specific HSEs. The hsp promoter has multiple copies of a conserved HSE containing contiguous inverted repeats of the 5-bp sequence nGAAn positioned upstream of the TATA box element (22). The transcription factor HSF is maintained in a monomeric form in the cytoplasm of nonstressed cells through direct binding to HSP-70. During cellular stress, HSP-70 binds to denatured protein and allows monomeric HSF to trimerize and migrate to the nucleus where it then binds to HSE. HSP transcription is initiated upon phosphorylation of the HSF trimer (22).

The hsp promoter has been used for over 10 years to selectively drive inducible expression of heterologous genes after hyperthermia (23-28). In the present study, we examined the effectiveness of PDT-mediated oxidative stress to initiate translation of heterologous genes ligated to the human hsp promoter. Clinically relevant photosensitizers were used in experiments designed to evaluate the efficiency of PDT to function as a molecular switch for the expression of reporter genes and cancer therapeutic genes in a selective and temporal manner.

## MATERIALS AND METHODS

**Photosensitizers.** PH was a gift from Quadra Logics, Inc. (Vancouver, British Columbia, Canada) and was dissolved in 5% dextrose in water to make a 2.5-mg/ml working solution. NPe6 was a gift from Porphyrin Products (Logan, UT) and was dissolved in saline at 2.5 mg/ml.

**Cell Lines.** Mouse RIF cells were originally obtained from G. Hahn (Stanford University, Palo Alto, CA) and were grown in RPMI 1640 medium supplemented with 15% FCS and antibiotics (29). Human ovarian adenocarcinoma (SKOV-3) cells were obtained from W. McBride (University of California, Los Angeles, Los Angeles, CA) and were grown in Dulbecco's minimal essential medium supplemented with 10% FCS and antibiotics (30). The SKOV-3 cells have a homozygously deleted *p53* gene (31) and exhibit resistance to recombinant *TNF*- $\alpha$  (32). Mouse fibrosarcoma (WEHI-13VAR) cells were obtained from American Type Culture Collection (Rockville, MD) and were grown in RPMI 1640 medium supplemented with 10% FCS. These

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<sup>3</sup> The abbreviations used are: PDT, photodynamic therapy; CAT, chloramphenicol acetyl transferase;  $\beta$ -gal,  $\beta$ -galactosidase; NPe6, mono-l-aspartyl chlorin e6; hsp, heat shock protein; PH, Photofrin porphyrin sodium; *TNF*, tumor necrosis factor; HSE, heat shock element; HSF, heat shock factor; EMSA, electrophoretic mobility shift assay; HBS, high binding site; RIF, radiation-induced fibrosarcoma; CMV, cytomegalovirus.



cells exhibit TNF- $\alpha$  sensitivity when treated in the presence of actinomycin D (33).

**Expression Vectors.** Plasmids p2500-CAT and p173OR (providing inducible expression of CAT or  $\beta$ -gal, respectively under the control of a 2.5-kb human hsp70 promoter fragment) were obtained from StressGen Biotech Corp. (Vancouver, British Columbia, Canada). Plasmid pMC1Neo (providing constitutive expression of the neomycin resistance gene under the control of the thymidine kinase promoter) was obtained from Stratagene (La Jolla, CA). Plasmid pCMV-neo-Bam-hp53 (providing constitutive expression of wt p53 under the control of the CMV promoter and G418 selectivity) was obtained from Y. Fung (Children's Hospital Los Angeles, Los Angeles, CA, Ref. 34). Plasmid pHSP.3hp53 (providing inducible expression of human p53 under the control of a human hsp promoter fragment together with G418 selectivity) was constructed by first removing the CMV promoter from pCMV-neo-Bam-hp53 and replacing it with a 0.3-kb fragment of the human hsp70 promoter from plasmid pD3SX, which was obtained from StressGen Biotech Corp. Plasmid pHSP.3hTNF (providing inducible expression of human TNF- $\alpha$  under the control of the 0.3-kb hsp promoter fragment together with G418 selectivity) was constructed by replacing p53 from pHSP.3hp53 with the 1.1-kb fragment of human TNF- $\alpha$  cDNA excised from pE4 (American Type Culture Collection). Plasmid p53-HBS was obtained from Y. Fung (Children's Hospital Los Angeles) and contains two copies of a 20-bp p53 HBS ligated upstream from a minimal thymidine kinase promoter linked to CAT. Reporter gene activation occurs when p53 binds to the HBS motif of this promoter (35). Plasmids were grown in supercompetent *Escherichia coli*, DH5- $\alpha$  (Life Technologies, Inc., Grand Island, NY), isolated, and purified using a Qiagen plasmid kit (Qiagen, Inc., Chatsworth, CA).

Reporter plasmids (p2500-CAT or p173OR) were transfected into RIF cells along with pMC1Neo (5:1 ratio) using calcium phosphate precipitation. Cells were grown in media containing 600  $\mu$ g/ml G418, and resulting colonies were picked using cloning rings. G418-resistant clones were expanded and tested for  $\beta$ -gal or CAT activity using heat (45°C for 20 min) as a positive inducing treatment. Individual clones exhibiting positive  $\beta$ -gal expression (HB-3) or CAT expression (HC-2) were isolated and used in subsequent studies. Expression plasmids containing inducible human genes (pHSP.3-hp53 or pHSP.3hTNF) were transfected into SKOV-3 cells using calcium phosphate precipitation. Cells were grown in media containing 800  $\mu$ g/ml G418, and resulting colonies were picked using cloning rings. G418-resistant clones were expanded and examined for p53 or TNF- $\alpha$  expression using heat (45°C for 20 min) as a positive inducer. Individual SKOV-3 cell clones exhibiting positive p53 expression (p53-S4) or TNF- $\alpha$  expression (TNF-S2) were isolated and used in subsequent studies.

**In Vitro PDT and Hyperthermia Treatments.** Photosensitization protocols involved seeding cells into plastic Petri dishes (60-mm dishes for survival analysis or 100-mm dishes for gene expression assays) and incubating overnight in complete growth media to allow for cell attachment. The plating efficiency for the parental RIF and transfected HC-2 and HB-3 cells ranged from 40 to 60%. Plating efficiencies for the parental SKOV-3 and transfected p53-S4 and TNF-S2 cells ranged from 50 to 70%. PDT treatments were performed as reported previously (13). Briefly, cells were incubated in the dark at 37°C for either 1 or 16 h with either PH or NPe6 (25  $\mu$ g/ml) in media containing either 1% or 5% FCS, respectively. Following the 1-h incubation protocol, cells were rinsed in media with serum and immediately exposed to graded doses of light. After the extended 16-h photosensitizer incubation, cells were rinsed for 30 min in growth media containing 15% FCS and then exposed to graded doses of light. Six hundred sixty-four-nm laser light delivered at a dose rate of 2 mW/cm<sup>2</sup> was used for cells incubated with NPe6. Broad spectrum red light (570–650 nm) generated by a parallel series of red millar-filtered 30-W fluorescent bulbs and delivered at a dose rate of 0.35 mW/cm<sup>2</sup> was used for cells incubated with Photofrin. Survival was measured using a standard clonogenic assay (29). *In vitro* hyperthermia involved seeding cells in T-25 or T-75 plastic flasks 24 h before exposure to warmed media and placement in a temperature-controlled water bath for specified time intervals (36). Inducible gene expression experiments were performed using PDT or hyperthermia treatments, which resulted in between 20 and 50% survival.

**In Vivo PDT and Hyperthermia Treatments.** Female C3H/HeJ mice (8–12 weeks old) were injected s.c. in the right hind flank with 10<sup>4</sup> parental RIF, HC-2, or HB-3 cells. Hyperthermia and PDT treatments were performed as reported previously on tumors measuring 6–7 mm in diameter and 3 mm in

height (37). Briefly, tumor hyperthermia involved a 20-min exposure to 810 nm of light emitted from an AlArO<sub>4</sub> diode laser at a dose rate of 220 mW/cm<sup>2</sup>. This procedure resulted in an intratumor temperature at a 1-mm depth of 44.5°C–45°C as measured with a 27-gauge needle thermister. PDT procedures included an i.v. injection of either PH or NPe6 at 5 mg/kg. Nonthermal PDT laser irradiation of tumors was initiated either 4 h (for NPe6) or 24 h (for PH) after photosensitizer administration. An argon-pumped dye laser emitted red light at 630 nm for PH-mediated PDT and 664 nm of light for NPe6-mediated PDT. A light dose rate of 75 mW/cm<sup>2</sup> was used for all *in vivo* PDT treatments. Total PDT light doses were 100 J/cm<sup>2</sup> for PH and 200 J/cm<sup>2</sup> for NPe6.

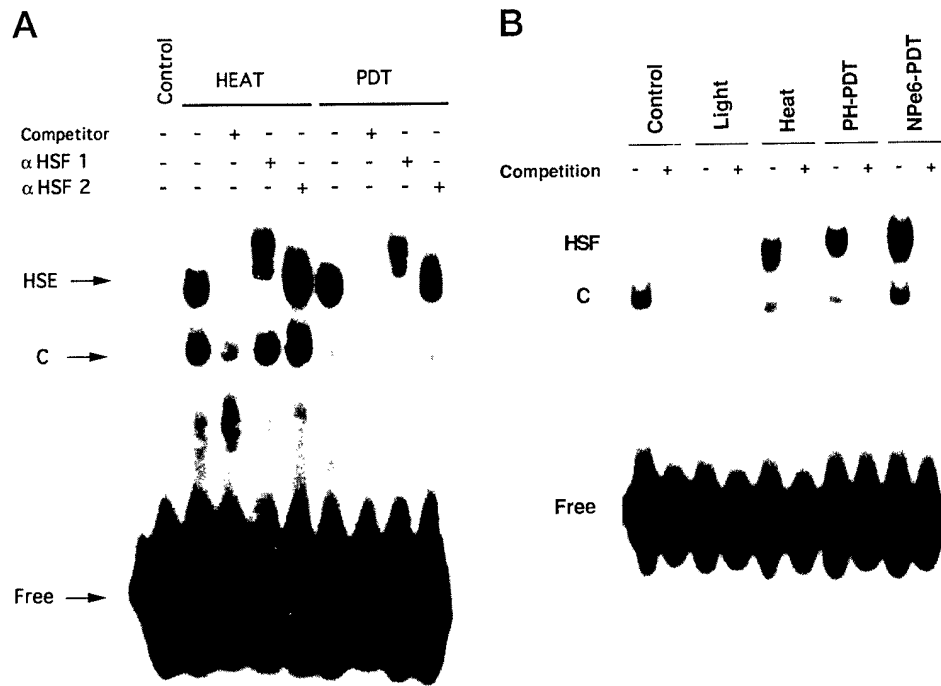
**Reporter Gene Assays.** Inducible expression of the *lacZ* gene product,  $\beta$ -gal, was evaluated in cells and tissue by photometric monitoring of the enzymatic cleavage of o-nitrophenyl-B-D-pyranogalactose (38). Briefly, treated cells were lysed in commercial Reporter Lysis Buffer (Promega, Madison, WI), scraped off the plastic dishes, and transferred to microfuge tubes. The suspension was centrifuged, and 150  $\mu$ l of supernatant were incubated for 3 h at 37°C with an equal volume of assay buffer containing o-nitrophenyl-B-D-pyranogalactose. Absorbance at 420 nm was determined for each sample, and  $\beta$ -gal activity (milliunits of  $\beta$ -gal per mg of protein) was calculated from a standard curve. For analysis of tumor tissue, samples were first homogenized with a Polytron in Reporter Lysis Buffer (40).  $\beta$ -gal activity was then determined as described above. CAT activity was determined by monitoring the transfer of the acetyl group from acetyl-CoA to <sup>14</sup>C-chloramphenicol using TLC (39). Briefly, treated cells were incubated with 1 ml of TEN solution [40 mM Tris (pH 7.5), 1 mM EDTA (pH 8.0), 150 mM NaCl], scraped off dishes, and concentrated by centrifugation. Cell pellets were lysed by freeze/thawing, and protein concentration was determined using a Bio-Rad protein assay. Cellular protein extracts were combined with acetyl-CoA solution and <sup>14</sup>C-chloramphenicol (40–60 mCi/mmol, ICN, Costa Mesa, CA) and incubated for 30 min at 37°C. Acetylated chloramphenicol was extracted in ethyl acetate and run on a silica gel TLC plate. CAT activity was determined by calculating the percent conversion to the acetylated forms of chloramphenicol.

**Western Blot Analysis.** Cells were collected in SDS lysing buffer at various times after treatment and evaluated for protein expression as described previously (16). Briefly, protein samples were size-separated on 10% polyacrylamide gels and transferred overnight to nitrocellulose membranes. Filters were blocked with 5% nonfat milk and then incubated with a mouse antihuman p53 monoclonal antibody (clone DO-1, Lab Vision Corp., Fremont, CA) or a mouse antiactin monoclonal antibody (clone C-4, ICN, Aurora, OH). Filters were then incubated with an antimouse peroxidase conjugate (Sigma, St. Louis, MO), and the resulting complex was visualized by enhanced chemiluminescence autoradiography.

**Mobility Shift Assays.** Tumor bearing mice with 6–8-mm diameter RIF tumors or RIF cells grown in culture were treated with either hyperthermia or PDT as described above. Binding of RIF-derived cellular protein to a mouse HSE was then analyzed with minor modifications of a previous procedure (18). Tumor samples were minced on ice and resuspended in extraction solution [20 mM HEPES (pH 7.9), 0.35 M NaCl, 20% glycerol, 1 mM MgCl<sub>2</sub>, 1% Nonidet, 1 mM DTT, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml aprotinin]. Samples were centrifuged, and supernatants were collected. A double-stranded 30-base oligonucleotide corresponding to the mouse HSE was 5' end-labeled with <sup>32</sup>P using a T4 polynucleotide kinase. Tumor-derived protein (10  $\mu$ g) was then added to a mixture containing 2  $\mu$ g of poly(dI-dC) and 10,000 cpm of <sup>32</sup>P-labeled HSE in 5  $\times$  binding buffer (18). For competitive analysis, 100-fold excess of nonradioactive HSE was added to duplicate reactions. Resulting DNA-protein complexes were resolved by electrophoresis on a 4% polyacrylamide gel. In supershift experiments involving the addition of antibodies to protein extracts before gel shift analysis, 0.2  $\mu$ g of a monoclonal antibody against HSF-1 or HSF-2 (Chemicon International, Inc., Temecula, CA) were added to the reaction mixture for 20 min at room temperature immediately before the addition of the radiolabeled HSE probe or cold competitor. After adding the probe, the reaction incubation was continued for 20 min at 37°C (41).

**ELISA Analysis of TNF- $\alpha$  Secretion.** A commercial TNF- $\alpha$  ELISA kit (Predicta, Genzyme Diagnostics, Cambridge, MA) was used for quantitative evaluation of TNF- $\alpha$  expression and secretion. Cells (10<sup>5</sup>) were seeded into 24-well plates, incubated overnight, and treated with either PDT or hyperther-

Fig. 1. Selective and inducible HSF DNA-binding activity occurs in RIF cells and tumors treated with hyperthermia or PDT. A, cells treated with hyperthermia or PDT exhibit specific HSF-1 binding to HSE. Whole cell extracts were collected 2 h after exposure to heat (45°C for 20 min) or 1 h after NPc6-mediated PDT (3400 J/m<sup>2</sup>). Protein extracts were incubated with either HSF-1 or HSF-2 antiserum for 20 min. Reaction mixtures were then incubated with a <sup>32</sup>P-labeled 30-bp mouse HSE fragment for 20 min and subjected to EMSA. Lanes marked *Competitor* were incubated with a 100-fold excess of nonradioactive HSE oligonucleotide; *C* indicates constitutive HSE binding activity; and *Free* indicates nonreactive mixtures. B, EMSA showing specific HSE binding activity of protein extracts from RIF tumors treated with either hyperthermia (45°C for 20 min), NPc6-mediated PDT (100 J/cm<sup>2</sup>), or PH-mediated PDT (100 J/cm<sup>2</sup>). Nontreated tumors and tumors exposed to only light served as controls. Mice were sacrificed 2 h after treatment, and tumor tissue was immediately collected and processed.



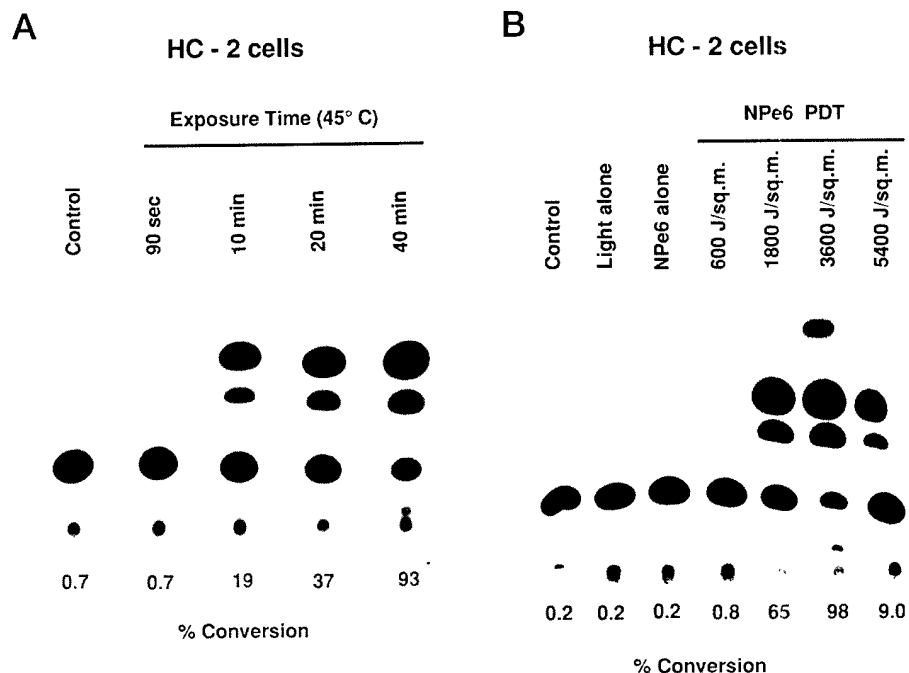
media as described above. Culture media was collected 24 h after treatment and analyzed for TNF- $\alpha$  according to the manufacturer's instructions.

**TNF- $\alpha$  Bioassay Analysis.** The biological activity of secreted TNF- $\alpha$  was evaluated by measuring cytotoxicity in TNF- $\alpha$  sensitive WEHI-13VAR cells (33). Cells were seeded in 96-well plates at a density of  $2 \times 10^4$  cells/well and incubated overnight. PDT and hyperthermia treatments were performed on SKOV-3 parental and TNF-S2 clones as described above, and media from these cells was collected 22 h after treatment when secreted TNF- $\alpha$  levels were found to be highest. The TNF- $\alpha$ -containing media was added to the WEHI-13VAR cells along with 0.5  $\mu$ g/ml actinomycin. Twenty four h later, the WEHI-13VAR cells were evaluated for cytotoxicity using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay from Chemicon Inc. (Temeccula, CA).

## RESULTS

**Specific HSF Binding to HSE Is Observed Following *in Vitro* and *in Vivo* PDT.** Activation of HSF involves the trimerization of monomeric HSF moieties followed by nuclear translocation and binding to an evolutionary conserved HSE (22). We reported previously that *in vitro* PDT can activate HSF in mammalian cells, but at that time, we did not identify the actual species of HSF (18). In the present study, we used murine RIF tumor cells and an electrophoretic gel mobility shift assay combined with HSF-1- and HSF-2-specific antiserum to determine which transcription factor(s) were activated by PDT. Fig. 1A shows that after PDT (using a 16-h cellular incubation

Fig. 2. Heat and PDT induce selective expression of CAT in RIF HC-2 cells stably transfected with the hsp promoter-controlled p2500-CAT plasmid. CAT activity in transfected HC-2 cells treated with increasing doses of (A) heat (45°C for times ranging from 1.5 to 40 min) or (B) NPc6-mediated PDT (25  $\mu$ g/ml NPc6 for 16 h; 600-5400 J/m<sup>2</sup>). Light alone and photosensitizer alone conditions did not induce CAT expression. Protein samples were collected 24 h after treatment. Conversion of chloramphenicol to acetylated chloramphenicol was calculated by counting radioactivity from resulting TLC plates.



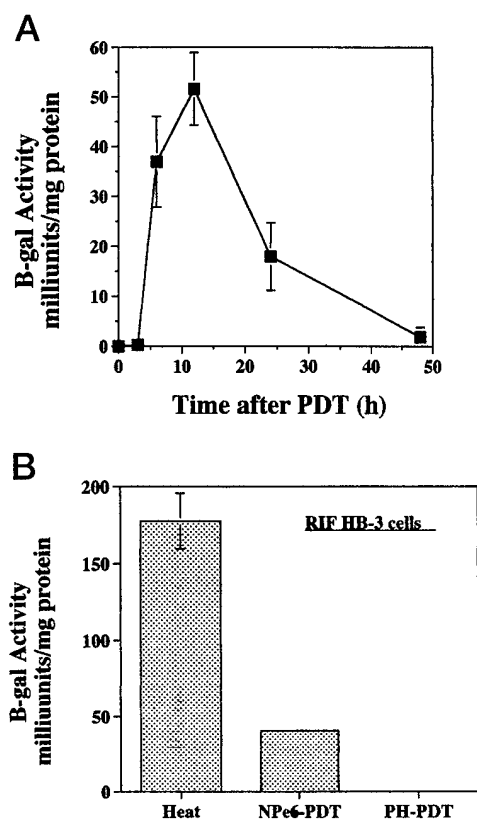


Fig. 3. Temporal- and photosensitizer-specific expression of the  $\beta$ -gal reporter gene occurs in RIF HB-3 cells stably transfected with the hsp promoter-controlled p173OR plasmid. **A**, kinetics of  $\beta$ -gal expression in RIF HB-3 cells treated with NPe6-PDT (16-h drug incubation; 3000 J/m<sup>2</sup>). Minimal enzyme activity was detected 3 h after treatment. Peak  $\beta$ -gal activity was observed 6 and 12 h after NPe6-mediated PDT.  $\beta$ -gal expression was not detected after light alone or photosensitizer alone conditions. Each data point represents the mean of at least three separate experiments  $\pm$  SE. **B**, differential expression of  $\beta$ -gal is observed in HB-3 cells exposed to isoeffective doses (inducing 15–25% survival) of heat, NPe6 PDT, or PH PDT. Treated cells were assayed for  $\beta$ -gal 6 h after treatment. Levels are the mean  $\pm$  SE from four to five separate experiments.

with NPe6), a supershift in the EMSA occurs in the presence of HSF-1 antibody but not in the presence of HSF-2. Treatment of RIF cells with PH and NPe6-mediated PDT (1 h photosensitizer incubation) also induced a positive but weaker supershift with HSF-1 (data not shown). Heat was used as a positive control and induced selective activation of HSF-1, which agrees with previous studies (21). Positive EMSA and supershift assays were not obtained after isoeffective *in vitro* PDT when a 16-h PH incubation was examined (data not shown). The ability of PDT to induce selective HSF binding to HSE was next examined under *in vivo* treatment conditions. Fig. 1B shows a representative EMSA for cellular protein extracted from RIF tumors growing in C3H mice. Protein from control tumors did not elicit selective HSE binding. However, protein extracted from tumors treated with either PH- or NPe6-mediated PDT produced extensive HSE binding, which could be competed away with cold HSE. Diode laser-generated tumor hyperthermia served as a positive control. These results indicate that PDT can induce both *in vitro* and *in vivo* HSF binding, which would be essential for PDT to transcriptionally activate heterologous genes under the control of an hsp promoter.

**PDT and Hyperthermia Induce Selective and Dose-dependent Expression of CAT Under the Control of a 2.5-kb hsp Promoter Fragment.** Stable integration of reporter gene expression vectors containing an inducible hsp70 promoter was achieved in RIF cells. Sensitivity of parental RIF and transfected HC-2 and HB-3 cells to either PDT or hyperthermia was similar (data not shown). This indi-

cates that transfection procedures and integration of heterologous DNA into mammalian cells does not modulate photosensitivity or thermal sensitivity. Fig. 2A shows that HC-2 cells exposed to 45°C expressed a dose-dependent increase in CAT activity. A similar pattern of induced CAT expression was documented in HC-2 cells exposed to increasing doses of NPe6-mediated PDT as shown in Fig. 2B. Inducible expression occurred when a 16-h NPe6 incubation was used. CAT expression was not initiated by either light alone or photosensitizer alone, indicating that the induction was the sole result of PDT-mediated oxidative stress. CAT expression initially increased with increasing PDT doses and then decreased as a greater percentage of cells were killed by the treatment. A reduction in CAT expression at increasingly lethal hyperthermia doses was also observed (data not shown). These results provide the first demonstration that PDT-mediated oxidative stress can activate a transgene under the control of an hsp promoter.

**PDT-mediated Expression of hsp Promoter-inducible Reporter Genes Is Transient As Well as Photosensitizer- and Incubation-specific.** High level expression of  $\beta$ -gal was observed in RIF HB-3 cells treated with either hyperthermia or NPe6-mediated PDT. Fig. 3A shows the kinetics of  $\beta$ -gal expression in HB-3 cells incubated for 16 h with NPe6 and then exposed to a 3000-J/m<sup>2</sup> light dose.  $\beta$ -gal activity was detected within 6 h of PDT treatment and continued to increase for at least 12 h before declining to background levels by 48 h after PDT. Similar kinetics of  $\beta$ -gal expression were observed following an isoeffective hyperthermia treatment (data not shown). Interestingly, the ability of PDT to induce  $\beta$ -gal or CAT expression in transfected RIF cells cultured *in vitro* was strongly dependent on the specific photosensitizer and incubation conditions being evaluated. Fig. 3B shows  $\beta$ -gal expression in HB-3 cells at 6 h after exposure to either heat or PDT. Maximal reporter gene expression was observed after hyperthermia. Significant  $\beta$ -gal expression was also observed in cells treated with NPe6 PDT (using a 16-h photosensitizer incubation protocol). A 16-h PH incubation before *in vitro* PDT resulted in minimally detectable  $\beta$ -gal expression. Likewise,  $\beta$ -gal expression was not detected in transfected RIF cells incubated for 1 h with either NPe6 or PH before light exposure (data not shown). Interestingly, different results were obtained when the transfected RIF cells were grown as solid tumors in C3H mice and treated with PDT (as described below).

***In Vivo* PDT Induces hsp Promoter-directed Reporter Gene Expression.** The stable integration of reporter gene constructs in RIF cells provided an opportunity to evaluate the ability of the hsp promoter to function under *in vivo* oxidative stress treatment parameters.

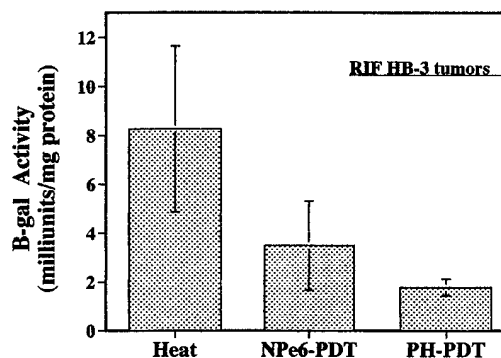


Fig. 4. NPe6- and PH-mediated PDT and laser-generated hyperthermia induce hsp promoter-directed  $\beta$ -gal expression in RIF HB-3 tumors growing in C3H/HeJ mice. Tumor samples were collected 16 h after PDT and heat treatments (as described in "Materials and Methods").  $\beta$ -gal measurements represent the mean  $\pm$  SE from five individual tumors.  $\beta$ -gal activity was not detected in nontreated tumors or in tumors treated with light or photosensitizer alone (data not shown).

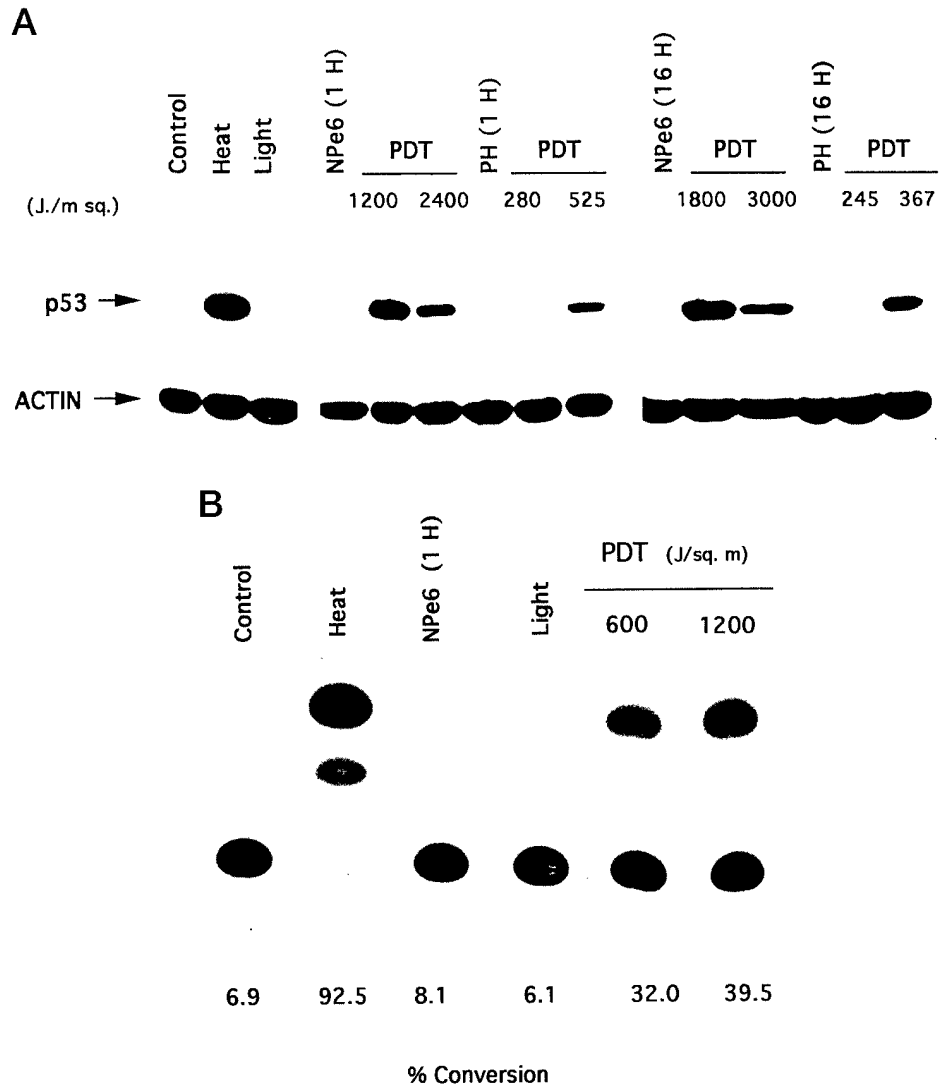


Fig. 5. PDT and heat induce selective expression of a functional human p53 protein in p53 null SKOV-3 cells stably transfected with the hsp promoter-controlled pHSP.3hp53 plasmid. **A**, Western immunoblots are shown for the parental SKOV-3 cells (control) and p53-S4 cells exposed to heat (45°C for 20 min) or various PDT treatments involving short (1 h) or extended (16 h) NPe6 and PH incubations prior to light. p53 expression was analyzed 6 h after treatment. The p53 blots were reprobed for actin protein levels as an indicator of sample loading. **B**, p53 induced by heat or PDT functions as a transcription factor. p53-dependent CAT expression is observed in p53-S4 cells transiently transfected with the p53-HBS reporter plasmid and exposed to either heat or NPe6-mediated PDT. CAT expression occurs when functional p53 binds to the p53-specific HBS motif of a minimal thymidine kinase promoter. Protein samples were collected 24 h after heat or PDT. Nontreated controls as well as light alone and photosensitizer alone conditions exhibited background CAT expression. Conversion of chloramphenicol to acetylated chloramphenicol was calculated by counting radioactivity from resulting TLC plates.

s.c. injection of parental RIF cells as well as HC-2 and HB-3 cells into the hind flank of C3H mice resulted in the reproducible formation of solid tumors amenable to laser hyperthermia or PDT treatment. Fig. 4 shows  $\beta$ -gal expression levels in tumors treated with either heat or PDT. Laser-induced hyperthermia produced a significant expression of  $\beta$ -gal, which was in agreement with *in vitro* data. *In vivo* PDT also functioned as an efficient molecular switch for inducible expression of  $\beta$ -gal in exposed tumor tissue. Both NPe6 and PH were equally capable of eliciting PDT-induced  $\beta$ -gal expression, although only NPe6 was capable of eliciting a significant *in vitro* response in RIF cells.

**PDT Induces Functional p53 Expression in Transfected SKOV-3 Cells.** Heat and PDT-inducible expression of p53 was evaluated in p53 null SKOV-3 cells stably transfected with pHSP.3p53 (p53-S4 cells). p53 protein expression was observed within 6 h of heat or PDT treatment and was maximal at 24 h (data not shown). The p53 expression decreased with extended times and dissipated by 72 h. Fig. 5A shows p53 protein expression assayed 6 h after cells were treated with either heat, PH-mediated PDT, or NPe6-mediated PDT. We conclude that PDT-mediated oxidative stress was responsible for inducing p53 expression because neither photosensitizer alone nor light alone induced any p53. Promoter leakiness was not observed in p53-S4 cells because p53 expression was not detected under control

conditions. The functionality of induced expression of p53 was determined using a transactivation reporter gene assay (38, 41). Fig. 5B shows p53-mediated CAT expression in p53-S4 cells treated with either heat or PDT. CAT expression was observed 24 h after both heat and NPe6-mediated PDT in transfected cells, indicating that expressed p53, documented by Western analysis in Fig. 5A, also functioned efficiently as a transcription factor. Comparable results were also observed for cells exposed to PDT using both 1-h and 16-h PH incubations (data not shown).

**Biologically Active TNF- $\alpha$  Is Secreted from SKOV-3 TNF-S4 Cells Following PDT and Hyperthermia.** The use of TNF- $\alpha$  in gene expression studies requires that the expressed cytokine can also be secreted from transfected cells. Fig. 6, A and B show that inducible secretion of biologically active TNF- $\alpha$  was achieved in TNF-S2 cells exposed to hyperthermia or PDT. The figures show levels of either heat or NPe6 PDT-induced TNF- $\alpha$  detected in the media of cultured TNF-S2 cells 24 h after treatment. The figures also show concomitant biological activity of the secreted cytokine. TNF- $\alpha$  was not detected in the culture media of parental SKOV-3 cells under any treatment conditions. However, a measurable and reproducible level of secreted TNF- $\alpha$  (23 pg/ml) was routinely obtained in transfected TNF-S2 cells under basal conditions. This indicates that hsp promoter leakiness occurred in our transfected human TNF-S2 cells. Fig. 6A shows that

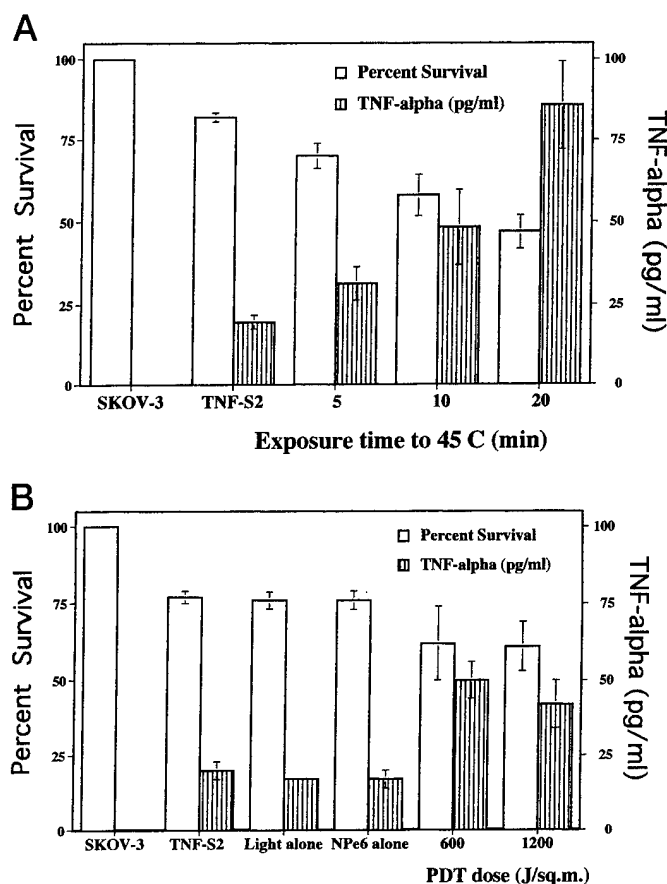


Fig. 6. PDT and hyperthermia induce expression and secretion of biologically active TNF- $\alpha$  in SKOV-3 cells stably transfected with the hsp promoter-controlled pHSP.3hTNF plasmid. Levels of TNF- $\alpha$  secreted into the culture media and concomitant sensitivity of WEHI-13 VAR cells exposed to this media are shown for TNF-S2 cells treated with either heat (A) or NPe6-mediated PDT (B). Treatment conditions included: heat (45°C for 5, 10, or 20 min) or PDT (1 h of NPe6 incubation followed by exposure to 600 or 1200 J/m<sup>2</sup> of 664-nm light). Supernatants were collected 22 h after treatment and either assayed for TNF- $\alpha$  using an ELISA kit or added to the culture media of WEHI-13VAR cells in the presence of 0.5  $\mu$ g/ml actinomycin. Cell survival was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 24 h after adding the TNF- $\alpha$  containing media. Each point represents the mean  $\pm$  SE from three separate experiments. Nontreated TNF-S2 cells as well as cells exposed to photosensitizer alone or light alone exhibit a detectable level of biologically active TNF- $\alpha$  indicative of promoter leakiness and constitutive background expression.

increasing heat exposure times resulted in an elevation in secreted TNF- $\alpha$  levels. Biological activity of the secreted TNF- $\alpha$  was determined using WEHI-13VAR cells. These cells provide a sensitive, reliable, and stable bioassay system for measuring TNF- $\alpha$  activity (33). Increased cell sensitivity was observed after incubation with media containing elevated TNF- $\alpha$  concentrations. Fig. 6B shows TNF- $\alpha$  secretion and concomitant biological activity for TNF-S2 cells exposed to NPe6-mediated PDT using a 1 h photosensitizer incubation protocol. Comparable induction of TNF- $\alpha$  was obtained using a 16-h NPe6 incubation PDT protocol as well as following both 1- and 16-h Photofrin incubation PDT protocols (data not shown). TNF-S2 cells exhibited basal expression of TNF- $\alpha$ , but exposure to photosensitizer alone or light alone did not activate the hsp promoter because TNF- $\alpha$  expression levels were comparable to levels for nontreated TNF-S2 cells.

## DISCUSSION

A primary goal of our study was to determine whether PDT could function as a molecular switch for controlling the expression of

heterologous genes. Clinically directed gene therapy uses expression constructs to replace/modify defective genes or to introduce genes encoding cytotoxic proteins or immunomodulators (42). However, the translation of gene therapy objectives into actual clinical practice requires overcoming a number of obstacles. These challenges include developing reproducible procedures for the efficient and safe delivery of DNA expression vectors to cells and tissues (43). For our study, we chose to focus on evaluating an inducible promoter approach for PDT-controlled activation of gene expression. A variety of inducible expression strategies are presently being examined in the context of localized gene therapy. Constructs responsive to ionizing radiation (using the *egr-1* promoter), hyperthermia (using the *hsp-70* promoter), and hypoxia (using the *grp-78* promoter or a hypoxia responsive element) are being tested for selective expression of therapeutic genes (28, 44–46). We hypothesized that the hsp promoter could be exploited for coupling the oxidative effects of PDT to an inducible procedure for expressing heterologous genes. Our results confirmed that PDT initiates HSF-1 binding to HSE in RIF cells incubated with NPe6. This is a necessary first step in using the hsp promoter with PDT for inducible transgene expression. We are unclear as to why differential binding was observed after PDT using NPe6 *versus* PH, but it may involve different subcellular targets (18). Interestingly, HSF binding to HSE and reporter gene expression occurred with both PH- and NPe6-mediated PDT when RIF cells were grown as solid tumors in mouse. *In vivo* PDT treatment elicits a pronounced inflammatory response involving the release of vasoactive and inflammatory mediators as well as the accumulation of host cells (4, 10). In this regard, prostaglandins activate HSF in mammalian cells and result in a thermotolerant state (47). Therefore, secondary physiological responses in PDT-treated tumor tissue may play a role in hsp promoter-controlled expression.

A variable PDT-induced gene expression profile was observed for porphyrin and chlorin photosensitizers in RIF cells. Similar differences were not observed in SKOV cells. The variable results detected in mouse *versus* human cells suggest that care must be taken in extrapolating preclinical studies to clinical PDT. These observations also agree with reports indicating that generalizations of PDT-induced biochemical and molecular pathways should not be made because responses vary depending on the specific photosensitizers and/or treatment parameters (2, 11). The effectiveness of the PDT-mediated molecular switch did not always follow HSF-HSE binding patterns. In RIF cells, PDT using a 16-h NPe6 incubation resulted in selective binding of HSF-1 to HSE. This treatment protocol also produced selective CAT and B-gal expression. Conversely, PH-mediated PDT did not elicit reporter gene expression in RIF cells but could induce HSF-HSE binding. Because both PDT procedures are associated with singlet oxygen-mediated oxidative stress, we conclude that distinct subcellular targets or molecular pathways may contribute to the differential results (48, 49). Additionally, a conserved 76-amino acid protein HSF binding protein has been identified in mammalian cells (50). This protein negatively effects HSF-1 DNA binding activity, and overexpression of this protein represses the transactivation activity of HSF-1. Likewise, overexpression of *bcl-2* suppresses transcriptional activation of *hsp70* (51). Various photosensitizers may have different effects on the expression of these molecules, but this still needs to be verified.

We selected two therapeutically relevant human genes, *p53* and *TNF- $\alpha$* , for initial analysis of our PDT-responsive molecular switch. These genes exhibit different biological functions and expression properties. *p53* encodes a tumor suppressor protein and functions as a transcription factor (52). This protein is mutated or deleted in numerous solid cancers, and overexpression of wild-type *p53* can enhance the therapeutic response of some malignancies to chemotherapy and

ionizing radiation (53). The significance of *p53* expression on PDT sensitivity has recently been examined, and responses appear to vary with cell type (38, 54). *p53* exerts its activity as a transcription factor within the same cells in which the protein is expressed. Our results confirmed that all examined PDT exposure parameters were able to selectively induce the transient expression of biologically active *p53* in *p53*-S4 cells. Parental SKOV cells are *p53* null, and therefore, we can conclude that *p53* expression in the transfected S4 cells was a direct consequence of PDT. Background expression or promoter leakiness was not observed in *p53*-S4 cells. Likewise, we were unable to detect *p53* expression when cells were exposed to photosensitizer or light alone.

In contrast to *p53*, *TNF- $\alpha$*  must first be secreted from producer cells before eliciting biological activity on cells with *TNF*-specific receptors (55). This cytokine is measurable in culture media with an ELISA assay, and the biological activity of *TNF- $\alpha$*  in this media can be monitored using a WEHI cell sensitivity assay (33). Systemically administered *TNF- $\alpha$*  enhances the cytotoxic effectiveness of therapeutic agents, including ionizing radiation and PDT (56, 57). However, toxicity associated with systemic *TNF- $\alpha$*  administration precludes its use as a therapeutic adjuvant. Inducible expression of *TNF- $\alpha$*  within target tissue or cells provides for local concentrations of the cytokine with minimal systemic effects. We documented that PDT exposure parameters can induce transient expression of biologically active *TNF- $\alpha$*  in *TNF*-S2 cells. Photofrin-mediated PDT produces a dose-dependent increase in *TNF- $\alpha$*  expression in peritoneal macrophages in treated mice (58). *TNF- $\alpha$*  is also detected in urine of patients undergoing local PDT for bladder cancer (59). However, we did not detect *TNF- $\alpha$*  in nontransfected SKOV tumor cells treated with PDT or hyperthermia. Our results indicate that the hsp promoter vigorously drives transgene expression. Secreted cytokine levels induced by PDT or heat in *TNF*-S2 cells were comparable or higher than *TNF- $\alpha$*  levels previously reported for constitutive expression systems driven by a CMV enhancer and B-actin promoter (60).

Background or basal expression of genes ligated to the hsp promoter was variable. Expression constructs transfected in RIF cells included a 2.5-kb fragment of the hsp promoter, whereas a 0.3-kb fragment of the hsp promoter was used with human SKOV-3 cells. Neither CAT nor  $\beta$ -gal expression was detected in RIF HC-2 and HB-3 cells under control conditions. Likewise, we did not observe any background leakiness of *p53* expression in *p53*-S4 cells. However, promoter leakiness was observed in *TNF*-S2 cells with a constant basal expression and secretion of *TNF- $\alpha$* . Therefore, hsp promoter size does not appear to be a primary determinant modulating transgene leakiness. However, vector insertion site could be involved in the differences we observed in basal expression. There are also three binding sites within the hsp promoter for the SP1 transcription factor, which may contribute to basal expression under various conditions (61). In addition, the hsp promoter contains a variety of regulatory elements, including AP-2, SRE, and c-myc binding sites (61). These elements can effect basal hsp promoter activity and can vary as a function of cell type and culture conditions.

In summary, PDT continues to show promise in the clinical treatment of solid malignancies. Nevertheless, methods to enhance the local tumoricidal responsiveness of PDT are still important because tumor recurrences are observed. We demonstrate for the first time that PDT-mediated oxidative stress can effectively induce selective expression of heterologous genes placed under the control of an hsp promoter. These results extend previous findings showing that PDT functions at the level of transcription to activate stress proteins (18). Our investigation provides initial proof of principle results indicating that PDT-responsive expression vectors should be examined in subsequent preclinical studies combining PDT and gene therapy.

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